IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent Application of:		**Docket No.:**	**4258-116**
Applicants:	**ALONSO FERNANDEZ, Maria Jose, et al.**	**Conf. No.:**	**9386**
Application No.:	**10/561,548**	**Art Unit:**	**1618**
Date Filed:	**March 7, 2006**	**Examiner:**	**Nissa M. Westerberg**
Title:	**HYALURONIC ACID NANOPARTICLES**	**Customer No.:**	**23448**

DECLARATION UNDER 37 CFR 1.132 OF ANNA ISABEL VILA PENA IN U.S. PATENT APPLICATION NO. 10/561,548

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, ANNA ISABEL VILA PENA, hereby declare:

1. THAT I am an employee of **ADVANCED IN VITRO CELL TECHNOLOGIES, S.L.**, of Barcelona, Spain (hereafter referred to as "AIVCT"), having a PhD degree in chemistry, and working in the laboratory of such company.

2. THAT I have been requested to conduct experimental work and to provide testimonial evidence in support of United States Patent Application No. 10/561,548 filed March 7, 2006 in the United States Patent and Trademark Office for "HYALURONIC ACID NANOPARTICLES" (hereafter referred to as the "Application"), and assigned to AIVCT.

3. THAT I am aware that the Application discloses and claims, as currently amended, nanoparticles as specified in the following claims 16, 17, 32 and 33 of the Application:

16. Nanoparticles with a diameter less than 1μm, characterized by a stability of at least one month at ambient temperature storage, for the administration of an active ingredient, which are obtained by a method comprising (a) preparing an aqueous solution of a hyaluronic acid salt, (b) preparing an aqueous solution of a cationic polymer, (c) adding sodium tripolyphosphate to the solution of the hyaluronic acid salt, (d) stir-mixing the solutions resulting from steps (b) and (c), spontaneously obtaining the nanoparticles, wherein the active ingredient is dissolved in one of resulting solutions (a), (b) or (c) or in a suspension of the nanoparticles obtained in step (d), to be absorbed in the nanoparticles, wherein the nanoparticles have no covalent bonds between the hyaluronic acid salt, cationic polymer, sodium tripolyphosphate and active ingredient.

17. Nanoparticles for the administration of an active ingredient, characterized by a stability of at least one month at ambient temperature, comprising a hyaluronic acid salt, a cationic polymer, sodium tripolyphosphate and the active ingredient as components, without covalent bonds between the components.

32. Nanoparticles according to claim 16, storage stable in particle size and zeta potential for at least one month.

33. Nanoparticles according to claim 17, storage stable in particle size and zeta potential for at least one month.

4. THAT I am aware that the Application at page 10, lines 23 to page 11, line 3 discloses a process for making the nanoparticles of the aforementioned claims of the Application, as follows:

"According to a first aspect, the present invention relates to a method of obtaining hyaluronic acid nanoparticles with a diameter less than 1μm, which incorporate an active ingredient, irrespective of the hydrophobic or hydrophilic nature thereof. This method comprises the following steps:
a) preparing an aqueous solution of a hyaluronic acid salt, preferably in a concentration of between 0.50 and 5 mg/mL;
b) preparing an aqueous solution of a cationic polymer, preferably in a concentration of between 0.50 and 5 mg/mL;
c) adding a polyanionic salt to the solution of the hyaluronic acid salt, preferably in a concentration of between 0.25 and 1.00 mg/mL;
d) stir-mixing the solutions resulting from steps b) and c), spontaneously obtaining the nanoparticles"

and that the Application at page 15, lines 9-17 discloses a process for making the nanoparticles of the aforementioned claims, as follows:

"Example 1
Hyaluronic acid nanoparticles in the form of sodium salt, chitosan as cationic polymer and sodium triphosphate as crosslinking agent, were prepared according to the previously described method. The hyaluronate and sodium triphosphate solution were added to the chitosan solution, with magnetic stirring, which is maintained for half an hour, permitting the complete evolution of the system towards a stable nanoparticulate form."

5. THAT I conducted the procedure of Example 1 of the Application, and observed that upon addition of hyaluronic acid salt/sodium triphosphate solution to the chitosan solution, and stir mixing of the solutions, nanoparticles were spontaneously formed, without the occurrence of gellation of the mixed solutions.

6. THAT the method of production of nanoparticles by the procedure of the Application as described in the above paragraphs 4 and 5 hereof was demonstrated not to require an emulsion formation since both solutions, the hyaluronic acid salt/sodium triphosphate solution as well as the chitosan solution, were aqueous solutions.

7. THAT the method of production of nanoparticles by the procedure of the Application as described in the above paragraphs 4 and 5 hereof was demonstrated not to require dropwise addition of one of solutions, namely, one of the hyaluronic acid salt/sodium triphosphate solution and the chitosan solution, into the other of such solutions.

8. THAT the method of production of nanoparticles by the procedure of the Application as described in the above paragraphs 4 and 5 hereof is consistent with an electrostatic interaction between the positively charged cationic polymer (chitosan) and the deprotonated form of hyaluronic acid, and, at the same time, the presence of the polyanionic salt (sodium triphosphate) inducing ionic crosslinking of the cationic polymer, causing the observed spontaneous formation of nanoparticles.

9. THAT I carried out the method of production of nanoparticles by the procedure of the Application as described in the above paragraphs 4 and 5 hereof, using chitosan as the

cationic polymer, and I also carried out a corresponding method of production of nanoparticles in which cationic collagen was used as the cationic polymer in place of chitosan, and a corresponding method of production of nanoparticles in which gelatin was used in place of chitosan.

10. THAT the nanoparticles produced according to the methods described in above paragraph 9 were comparatively tested to determine their stability in terms of particle size (nm) and polydispersity index during storage for 4 weeks at 4°C, with the results shown in the folllowing Table 1, wherein the following abbreviations are used - HA: sodium hyaluronate; COL: cationic collagen; GEL: gelatin; CS: chitosan; P.I: polydispersity index; n.d: not determined.

Table 1. Nanoparticles stability in terms of particle size and polydispersity index during 4 weeks at 4°C

Formulation	Composition (mass ratio)	Stability at 4°C									
		0		1 week		2 weeks		3 weeks		4 weeks	
		Size	P.I	Size	P.I	Size	P.I	Size	P.I	Size	P.I
COL/HA	1.2/1	180 ± 4	0.1	238 ± 4	0.3	260 ± 3	0.5	217 ± 6	0.3	190 ± 4	0.3
	1.5/1	202 ± 4	0.1	288 ± 3	0.3	285 ± 6	0.2	237 ± 5	0.2	245 ± 19	0.2
	2/1	217 ± 2	0.1	404 ± 5	0.2	392 ± 15	0.1	427 ± 47	0.4	319 ± 13	0.2
GEL/HA	1/1	266 ± 7	0.1	301 ± 5	0.1	296 ± 6	0.1	n.d	n.d	329 ± 19	0.1
CS/HA	1/2	127 ± 1	0.1	126 ± 1	0.1	134 ± 3	0.1	138 ± 5	0.1	139 ± 2	0.1

HA: sodium hyaluronate; COL: cationic collagen; GEL: gelatin; CS: chitosan; P.I: polydispersity index; n.d: not determined

11. THAT the nanoparticles produced according to the methods described in above paragraph 9 were comparatively tested to determine their stability in terms of particle size (nm) and polydispersity index during storage for 4 weeks at 25°C, with the results shown in the following Table 2, wherein the following abbreviations are used - HA: sodium hyaluronate; COL: cationic collagen; GEL: gelatin; CS: chitosan; P.I: polydispersity index; n.d: not determined.

Table 2. Nanoparticles stability in terms of particle size and polydispersity index during 4 weeks at 25°C

Formulation	Composition (mass ratio)	Stability at 25°C									
		0		1 week		2 weeks		3 weeks		4 weeks	
		Size	P.I	Size	P.I	Size	P.I	Size	P.I	Size	P.I
COL/HA	1.2/1	180 ± 4	0.1	310 ± 13	0.2	282 ± 6	0.4	228 ± 5	0.3	224 ± 7	0.2
	1.5/1	202 ± 4	0.1	397 ± 11	0.3	322 ± 20	0.4	329 ± 29	0.2	289 ± 43	0.4
	2/1	217 ± 2	0.1	480 ± 11	0.1	436 ± 18	0.3	414 ± 13	0.3	353 ± 7	0.3
CS/HA	1/2	127 ± 1	0.1	135 ± 2	0.1	133 ± 1	0.1	146 ± 3	0.1	139 ± 3	0.1

HA: sodium hyaluronate; COL: cationic collagen; GEL: gelatin; CS: chitosan; P.I: polydispersity index

12. THAT the results set out in Tables 1 and 2 above show the nanoparticles prepared according to the method of the Application, using different cationic polymers, and subsequently stored at ambient temperatures of 4°C and 25°C, to be stable in terms of particle size and polydispersity index over the 4 weeks period of the test.

13. THAT the nanoparticles produced according to the methods described in above paragraph 9 were also comparatively tested to determine their stability in terms of zeta potential after storage for 4 weeks at 4°C, and after storage for 4 weeks at 25°C, with the results shown in the following Table 3, wherein the following abbreviations are used - HA: sodium hyaluronate; COL: cationic collagen; GEL: gelatin; CS: chitosan; and (-): aggregation and/or precipitation.

Table 3. Nanoparticles stability in terms of superficial charge (zeta potential) after 4 weeks at 4 and 25ºC.

Formulation	Composition (mass ratio)	Zeta Potential (mV)	
		4ºC	25ºC
COL/HA	1.2/1	-41 ± 5	-38 ± 5
	1.5/1	-26 ± 7	-39 ± 5
	2/1	-47 ± 6	-50 ± 4
GEL/HA	1/1	-26 ± 5	(-)
CS/HA	1/2	+18 ± 9	+16 ± 8

HA: sodium hyaluronate; COL: cationic collagen; CS: chitosan; GEL: gelatin; (-): aggregation and/or precipitation

14. THAT the results set out in Table 3 above show nanoparticles prepared according to the method of the Application to be stable after 4 weeks storage at the ambient temperatures shown in the Table.

As the below-named declarant, I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present application or any patent issued thereon.

9. 12. 2009

ANA ISABEL VILA PENA

DATE